

published under Publication Number WO 00/07545, published February 17, 2000. The newly submitted claim set is to replace the pending claims. All claims not reiterated are to be cancelled without prejudice or disclaimer.

IN THE SPECIFICATION:

Please replace the paragraph beginning at page 2, line 16, with the following rewritten paragraph:

- B¹
- According to the objects outlined above, the present invention provides recombinant nucleic acids encoding Apop proteins, and in particular Apop1, Apop2 and Apop3 proteins, that are at least about 85% identical to the amino acid sequence depicted in Figure 2, Figure 4, and Figure 6 (SEQ ID NOS:2, 4 & 6), respectively. Similarly provided are recombinant nucleic acids at least about 85% identical to the nucleic acid sequence depicted in Figures 1, 3, and 5 (SEQ ID NOS:1, 3 & 5) or their complements. Expression vectors and host cells comprising the nucleic acids are also included.–

Please replace the paragraph beginning at page 2, line 25, with the following rewritten paragraph:

- B²
- In an additional aspect, the invention provides recombinant Apop proteins, that are at least about 85% identical to the amino acid sequences depicted in Figures 2, 4, and 6 (SEQ ID NOS:2, 4 & 6), respectively, and antibodies that will bind to the Apop1 proteins, Apop2 proteins and Apop3 proteins.–

Please replace the paragraph beginning at page 3, line 16, with the following rewritten paragraph:

- B³
- In one aspect, a method for screening for a bioactive agent comprises providing a cell that expresses an expression profile gene selected from the group consisting of the expression profile genes set forth in Figures 1, 3, and 5 (SEQ ID NOS:1, 3 & 5).–

Please replace the paragraph beginning at page 3, line 19, with the following rewritten paragraph:

- B⁴
- In another aspect, a method for screening for a bioactive agent, comprises providing a cell that expresses an expression profile protein selected from the group consisting of the expression profile proteins having the amino acid sequence set forth in Figures 2, 4, and 6 (SEQ ID NOS:2, 4 & 6).–

Please replace the paragraph beginning at page 3, line 22, with the following rewritten paragraph:

- B⁵
- In another aspect, the invention provides a biochip comprising recombinant nucleic acids encoding Apop proteins. The recombinant nucleic acids, encoding Apop proteins and bound to the biochip, may be at least about 85% identical to the nucleic acid sequence depicted in Figures 1, 3, and 5 (SEQ ID NOS:1, 3 & 5) or their complements. –

Beginning at page 4, line 1, please replace the section titled "BRIEF DESCRIPTION OF THE DRAWINGS" with the following rewritten section:

– Figure 1 (SEQ ID NO:1) depicts the nucleotide sequence of human Apop1. The putative translation start codon (ATG) and translation termination codon (TGA) are underlined.

Figure 2 (SEQ ID NO:2) depicts the amino acid sequence of human Apop1.

Figure 3 (SEQ ID NO:3) depicts the nucleotide sequence of human Apop2. The putative translation start codon (ATG) and the translation termination codon (TGA) are underlined.

Figure 4 (SEQ ID NO:4) depicts the amino acid sequence of human Apop2.

Figure 5 (SEQ ID NO:5) depicts the nucleotide sequence of human Apop3.

Figure 6 (SEQ ID NO:6) depicts the amino acid sequence of human Apop3.

Figure 7 depicts a schematic representation of Apop3 truncation mutants. The shaded area depicts the kinase homology domain. Apop3(K50D) has a K to D mutation at amino acid 50. WT, wild-type Apop3, i.e., the full-length protein.

Figure 8 depicts a summary of binding results of Apop3 to RIP in the yeast two hybrid system. +++++, very strong interaction; +++, strong interaction; +, detectable interaction; -, no detectable interaction.

Figure 9 depicts the activation of apoptosis by Apop3 in Phoenix-A cells. Empty vector (Vector), Apop3, or Apop3 (1-436) (3 µg) was co-transfected with pGDB (1 µg) into Phoenix-A cells. Hoechst stained apoptotic Phoenix-A cells were examined and counted by fluorescence microscopy. The data are expressed as percentage of apoptotic cells among the total number of cells counted.

Figure 10 depicts the activation of NFkB by Apop3 in Phoenix-A cells. NFkB reporter activity was performed by transiently co-transfecting Phoenix-A cells with the indicated Apop3 expression vectors (3 µg), NFkB-dependent luciferase reporter plasmid (1 µg), and Renilla Luciferase expression vector (0.13 µg). Calcium phosphate precipitation method was used for transfection. The level of expressed tagged proteins was also monitored by Western blot analysis. –

Please replace the paragraph beginning at page 5, line 11, with the following rewritten paragraph:

B1

– Thus, the present invention provides novel apoptosis proteins, termed Apop proteins and nucleic acids encoding them. Unless otherwise explicitly stated herein, the terms "Apop", "Apop protein" or grammatical equivalents thereof include Apop1 proteins, Apop2 proteins, and Apop3 proteins, the wild-type amino acid sequences of which are depicted in Figure 2, Figure 4, and Figure 6 (SEQ ID NOS:2, 4 & 6), respectively. Similarly, the terms "Apop nucleic acid", "Apop DNA", "Apop nucleotide sequence" or grammatical equivalents thereof include nucleic acids which encode Apop1 proteins, Apop2 proteins and Apop3 proteins, the wild-type nucleic acid sequences of which are depicted in Figure 1, Figure 3, and Figure 5 (SEQ ID NOS:1, 3 & 5), respectively.—

Please replace the paragraph beginning at page 5, line 24, with the following rewritten paragraph:

B8

– An Apop protein of the present invention may be identified in several ways. "Protein" in this sense includes proteins, polypeptides, and peptides. An Apop nucleic acid or Apop protein is initially identified by substantial nucleic acid and/or amino acid sequence homology to the sequences shown in Figures 1, 2, 3, 4, 5, and 6 (SEQ ID NOS:1-6). Such homology can be based upon the overall nucleic acid or amino acid sequence.—

Please replace the paragraph beginning at page 5, line 29, with the following rewritten paragraph:

B9

– As used herein, a protein is an "Apop protein" if the overall homology of the protein sequence to the amino acid sequences shown in Figures 2, 4, and 6 (SEQ ID NOS:2, 4 & 6) is preferably greater than about 75%, more preferably greater than about 80%, even more preferably greater than about 85% and most preferably greater than 90%. In some embodiments the homology will be as high as about 93 to 95 or 98%.—

Please replace the paragraph beginning at page 7, line 1, with the following rewritten paragraph:

B10

– In a similar manner, "percent (%) nucleic acid sequence identity" with respect to the coding sequence of the polypeptides identified herein is defined as the percentage of nucleotide residues in a candidate sequence that are identical with the nucleotide residues in the coding sequence of the Apop proteins (see Figures 1, 3, and 5 (SEQ ID NOS:1, 3 & 5)). A preferred method utilizes the BLASTN module of WU-BLAST-2 set to the default parameters, with overlap span and overlap fraction set to 1 and 0.125, respectively.—

Please replace the paragraph beginning at page 7, line 11, with the following rewritten paragraph:

B11

– The alignment may include the introduction of gaps in the sequences to be aligned. In addition, for sequences which contain either more or fewer amino acids than the protein sequences shown

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in Figures 2, 4, and 6 (SEQ ID NOS:2, 4 & 6) it is understood that the percentage of homology will be determined based on the number of homologous amino acids in relation to the total number of amino acids. Thus, for example, homology of sequences shorter than that shown in Figures 2, 4, and 6, as discussed below, will be determined using the number of amino acids in the shorter sequence.—

Please replace the paragraph beginning at page 7, line 30, with the following rewritten paragraph:

B¹²

— In a preferred embodiment, the Apop protein is an Apop1 protein. The nucleotide sequence of Apop1 is depicted in Figure 1 (SEQ ID NO:1) and the encoded protein in Figure 2 (SEQ ID NO:2). A significant portion of the Apop1 nucleic acid and the encoded Apop1 protein has identity to human cathepsin B precursor protein and the corresponding DNA [Ritonja et al., FEBS Lett. 181:169-172 (1985); Chan et al., Proc Natl. Acad. Sci. U.S.A. 83:7721-7725 (1986); Fong et al., Proc Natl. Acad. Sci. U.S.A. 83:2909-2913 (1986); Moin et al., Biochem. J. 285:427-434 (1992); Cao et al., Gene 139:163-169 (1994)]. Cathepsin B is a lysosomal thiol proteinase that may have additional extralysosomal functions. In particular, nucleotides 364 to 716 of the Apop1 nucleotide sequence depicted in Figure 1 have >99% (352/353 nucleotide residues) identity to human cathepsin B (CTSB) mRNA (GenBank accession numbers NM_001908 and M14221). Likewise, on the amino acid level, the Apop1 amino acid sequence depicted in Figure 2, shows 100% (109/109 amino acid residues) identity to human cathepsin B precursor protein (GenBank accession number P07858) and >99% (108/109 amino acid residues) identity to human cathepsin B precursor protein (GenBank accession number NP_001899). However, the remainder of the Apop1 protein and nucleic acid do not exhibit any homology to the known sequences. Thus the Apop1 nucleic acid sequence of the present invention may represent an alternatively spliced cathepsin B mRNA transcript and the encoded protein may have other biological activities when compared to cathepsin B.—

Please replace the paragraph beginning at page 8, line 18, with the following rewritten paragraph:

B¹³

— In a preferred embodiment, the Apop protein is an Apop2 protein. The nucleotide sequence of Apop2 is depicted in Figure 3 (SEQ ID NO:3) and the encoded protein in Figure 4 (SEQ ID NO:4). Apop2 nucleic acid and the encoded Apop2 protein have identity to human cathepsin F precursor protein and the corresponding DNA [Wang et al., J. Biol. Chem. 273:32000-8 (1998); Nagler et al., Biochem. Biophys. Res. Commun. 257:313-8 (1999); Santamaria et al., J. Biol. Chem. 274:13800-9 (1999); Wex et al., Biochem. Biophys. Res. Commun. 259:401-7 (1999); GenBank accession numbers AF071748, NM_003793, AF088886, AJ007331, and AF132894]. Cathepsin F is a novel papain-like cysteine proteinase, synthesized as a precursor protein, including a hydrophobic signal sequence, a pro-domain, and a catalytic region [Santamaria et al., J. Biol. Chem. 274:13800-9 (1999)].

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Surprisingly, Apop2 has strong homology to several other cathepsins. Protein sequence comparison revealed 58% homology with cathepsin W; about 42-43% with cathepsin L, K, S, H, and O; and 38% with cathepsin B [Wang et al., J. Biol. Chem. 273:32000-8 (1998)]. The identification of Apop2 (cathepsin F) by binding to XIAP, represents the first time that a protein containing cathepsin homology has been shown to play a role in apoptosis.—

Please replace the paragraph beginning at page 9, line 4, with the following rewritten paragraph:

B'4

— In a preferred embodiment, the Apop protein is an Apop3 protein. Using RIP as the “bait protein” in a yeast two-hybrid screening, Apop3 was identified. The nucleotide sequence of Apop3 is depicted in Figure 5 (SEQ ID NO:5) and the encoded protein in Figure 6 (SEQ ID NO:6). The human Apop3 appears to be expressed in heart, liver, pancreas, placenta and lung, but either weakly or not at all in brain. Apop3 has also been termed “RIP3” [see Yu et al., Current Biology 9:539-42 (1999); Sun et al., J. Biol. Chem. 274:16871-5 (1999)]. The N-terminal portion of Apop3, in particular amino acid residues 1-274, has homology to the kinase domain of RIP (34% identity and 60% similarity) and RIP2 (31% identity and 58% similarity) [see Sun et al., J. Biol. Chem. 274:16871-5 (1999)]. However, the C-terminal portion of Apop3 has no significant homology to any known proteins. Accordingly, Apop3 proteins may be identified in one aspect by significant homology to areas other than the kinase domain. This homology is preferably greater than about 60%, with greater than about 70 or 75% being particularly preferred and greater than about 80% being especially preferred. In some cases the homology will be greater than about 90 to 95 or 98%.—

Please replace the paragraph beginning at page 9, line 21, with the following rewritten paragraph:

B'5

Apop proteins of the present invention may be shorter or longer than the amino acid sequences shown in Figures 2, 4, and 6 (SEQ ID NOS:2, 4 & 6). Thus, in a preferred embodiment, included within the definition of Apop proteins are portions or fragments of the sequences depicted herein. Portions or fragments of Apop proteins are considered Apop proteins if a) they share at least one antigenic epitope; or b) have at least the indicated homology; or c) preferably have Apop biological activity, e.g., including, but not limited to kinase activity, cell death activity, binding to XIAP or RIP, etc.—

Please replace the paragraph beginning at page 10, line 12, with the following rewritten paragraph:

B'6

— In addition, as is more fully outlined below, Apop proteins can be made that are longer than those depicted in Figures 2, 4, and 6 (SEQ ID NOS:2, 4 & 6), for example, by the addition of epitope or purification tags, the addition of other fusion sequences, etc.—

Please replace the paragraph beginning at page 10, line 15, with the following rewritten paragraph:

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– Apop proteins may also be identified as being encoded by Apop nucleic acids. Thus, Apop proteins are encoded by nucleic acids that will hybridize to the sequence depicted in Figures 1, 3, and 5 (SEQ ID NOS:1, 3 & 5) or its complement, as outlined herein.—

Please replace the paragraph beginning at page 10, line 22, with the following rewritten paragraph:

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– Apop antibodies usually are generated with an Apop protein having the amino acid sequence depicted in Figures 2, 4, and 6 (SEQ ID NOS:2, 4 & 6). In a preferred embodiment, Apop proteins corresponding to a portion or fragment of an Apop protein of which the amino acid sequence is depicted in Figures 2, 4, and 6, are used to generate antibodies. Methods for the preparation and purification of monoclonal and polyclonal antibodies are known in the art and e.g., are described in Harlow and Lane, *Antibodies: A Laboratory Manual* (New York: Cold Spring Harbor Laboratory Press, 1988). When the Apop protein is used to generate antibodies, the Apop protein must share at least one epitope or determinant with the full length protein shown in Figures 2, 4, and 6. By "epitope" or "determinant" herein is meant a portion of a protein which will generate and/or bind an antibody. Thus, in most instances, antibodies made to a smaller Apop3 protein will be able to bind to the full length protein.—

Please replace the paragraph beginning at page 11, line 12, with the following rewritten paragraph:

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– In a preferred embodiment, an Apop protein of the present invention may be identified by its immunological activity, i.e., its ability to bind to an antibody specific for an epitope found within a protein comprising the amino acid sequence depicted in Figures 2, 4, and 6 (SEQ ID NO:2, 4 & 6). The term "immunological activity" means the ability of the protein to cross react with an antibody which is specific for the protein comprising the amino acid sequence depicted in Figures 2, 4, and 6, i.e., an Apop protein antibody. Accordingly, a protein is an Apop protein, if the protein displays the immunological activity of a protein comprising the amino acid sequence depicted in Figures 2, 4, and 6. —

Please replace the paragraph beginning at page 13, line 15, with the following rewritten paragraph:

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– In a preferred embodiment, Apop nucleic acids are provided. An Apop nucleic acid of the present invention may be identified by its sequence identity to the nucleotide sequence depicted in Figures 1, 3, or 5 (SEQ ID NOS:1, 3 or 5) and may be referred to as having some "percent (%)" sequence identity to all or a portion of the nucleotide sequence depicted in Figures 1, 3, or 5. Sequence identity, when referring to nucleic acid, means that the sequences being compared have nucleotides at corresponding positions which are identical. The sequence identity of an Apop nucleic acid is

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commensurate with the sequence identity of Apop proteins but takes into account the degeneracy in the genetic code and codon bias of different organisms. Accordingly, the sequence identity for Apop nucleic acids may differ from the sequence identity for Apop proteins. Thus, the nucleic acid sequence homology may be either lower or higher than that of the protein sequence. Thus the homology of the nucleic acid sequence as compared to the nucleic acid sequences of Figures 1, 3, and 5 is preferably greater than 75%, more preferably greater than about 80%, particularly greater than about 85% and most preferably greater than 90%. In some embodiments the homology will be as high as about 93 to 95 or 98%.—

Please replace the paragraph beginning at page 14, line 1, with the following rewritten paragraph:

B²⁷

— In one embodiment, the nucleic acid homology is determined through hybridization studies. Thus, for example, nucleic acids which hybridize under high stringency to the nucleic acid sequences shown in Figures 1, 3, and 5 (SEQ ID NOS:1, 3 or 5) or their complements are considered an Apop gene. High stringency conditions are known in the art; see for example Sambrook et al., *Molecular Cloning: A Laboratory Manual* (New York: Cold Spring Harbor Laboratory Press, 1989) and Ausubel et al., *Short Protocols in Molecular Biology* (John Wiley & Sons, Inc., 1995), both of which are hereby incorporated by reference. Stringent conditions are sequence-dependent and will be different in different circumstances. Longer sequences hybridize specifically at higher temperatures. An extensive guide to the hybridization of nucleic acids is found in Tijssen, *Techniques in Biochemistry and Molecular Biology: Hybridization with Nucleic Acid Probes*, "Overview of principles of hybridization and the strategy of nucleic acid assays" (1993). Generally, stringent conditions are selected to be about 5-10°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength pH. The T_m is the temperature (under defined ionic strength, pH and nucleic acid concentration) at which 50% of the probes complementary to the target hybridize to the target sequence at equilibrium (as the target sequences are present in excess, at T_m , 50% of the probes are occupied at equilibrium). Stringent conditions will be those in which the salt concentration is less than about 1.0 sodium ion, typically about 0.01 to 1.0 M sodium ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30°C for short probes (e.g. 10 to 50 nucleotides) and at least about 60°C for long probes (e.g. greater than 50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide.—

Please replace the paragraph beginning at page 38, line 5, with the following rewritten paragraph:

B²²

— Apop sequences bound to biochips include both nucleic acid and amino acid sequences as defined above. In a preferred embodiment, nucleic acid probes to Apop nucleic acids (both the nucleic acid sequences having the sequences outlined in Figures 1, 3, and 5 (SEQ ID NOS:1, 3 & 5) and/or the

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complements thereof) are made. The nucleic acid probes attached to the biochip are designed to be substantially complementary to the Apop nucleic acids, i.e. the target sequence (either the target sequence of the sample or to other probe sequences, for example in sandwich assays), such that hybridization of the target sequence and the probes of the present invention occurs. As outlined below, this complementarity need not be perfect; there may be any number of base pair mismatches which will interfere with hybridization between the target sequence and the single stranded nucleic acids of the present invention. However, if the number of mutations is so great that no hybridization can occur under even the least stringent of hybridization conditions, the sequence is not a complementary target sequence. Thus, by "substantially complementary" herein is meant that the probes are sufficiently complementary to the target sequences to hybridize under normal reaction conditions, particularly high stringency conditions, as outlined herein.—

~~Please replace the paragraph beginning at page 42, line 25, with the following rewritten paragraph:~~

B23

— In another preferred embodiment, antisense RNAs and DNAs can be used as therapeutic agents for blocking the expression of Apop genes *in vivo*. The term "antisense" herein means that the nucleic acid sequence of the antisense RNA or DNA comprises the reverse complement sequence of the mRNA to which it can bind. For example, if the mRNA, whose expression (i.e., translation into a protein) is to be blocked, comprises the nucleic acid sequence 5'-GGAAUUGGAGC-3' (SEQ ID NO:7), then the antisense RNA comprises the nucleic acid sequence of 5'-GCUCCAAUUCC-3' (SEQ ID NO:8) and the antisense DNA comprises the nucleic acid sequence of 5'-GCTCCAATTCC-3' (SEQ ID NO:9).—

~~Please replace the paragraph beginning at page 44, line 16, with the following rewritten paragraph:~~

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— Several known TNF α signaling proteins have been shown to bind RIP [Hsu et al., Immunity 4:387-396 (1996)] and complexes formed by the interactions between RIP kinase, TRADD, FADD, and RAIDD recruit other proteins to TNFR1 or Fas receptors to initiate signaling [Stanger et al., Cell 81:513-523 (1995); Hsu et al., Immunity 4:387-396 (1996); Tartaglia et al., Cell 74:845-853 (1993); Darnay et al., J. Leukoc. Biol. 61:559-566 (1997); Ashkenazi and Dixit, Science 281:1305-1308 (1998)]. We established a large-scale yeast two hybrid screening system designed to isolate rare mRNAs and verify novel protein-protein interactions involved in the TNF signaling pathway. Using RIP as bait, we screened 96 million independent yeast transformants with a combined HeLa/Lymphocyte cDNA library in a single transformation round. As expected, many of the known RIP binding proteins, including TRAF1, TRAF2, TNFR1, RIP, TRADD, and FADD, were cloned; several novel genes were isolated as well. One of the novel cDNAs had high sequence homology with RIP. The full-length cDNA (Figure 5; SEQ ID NO:5) contains a 518 amino acid open reading frame, encoding a protein

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B24

designated Apop3. Analysis of the amino acid sequence of this novel protein (Figure 6; SEQ ID NO:6) revealed a kinase domain (aa 21-287) which had 47% homology to the kinase domain of RIP and 42% homology to that of RIP2/Rick/CARDIAK. However, outside the kinase domain, the C-terminal region of Apop3 had no homology to death domains [Tartaglia et al., Cell 74:845-853 (1993)], death effector domains [Siegel et al., J. Cell. Biol. 141:1243-1253 (1998)], or the CARD region [Hofmann et al., Trends Biochem. Sci. 22:155-156 (1997)]. An approximately 2.1 Kb transcript was detected with a cDNA probe specific to Apop3 in both normal human tissue and human tumor RNA blots (data not shown).—

Please replace the paragraph beginning at page 47, line 2, with the following rewritten paragraph:

B25

— TNF α and Fas ligand induced apoptosis is controlled by caspase activation [Martin and Green, Cell 82:349-352 (1995)]. Rick/RIP2/CARDIAK kinase has also been reported to induce apoptosis through enhancement of caspase activity [Inohara et al., J. Bio. Chem. 273: 12296-12300 (1998)]. To test whether Apop3 activates cellular caspases, pYCI-Apop3 (data not shown) was co-transfected into Phoenix-A cells with pGDB. In this example, 2×10^6 Phoenix-A cells were co-transfected with expression vectors encoding Apop3 and Apop3 mutants, such as depicted in Figure 7 (10 μ g) with pGDB (3 μ g). Lysates were analyzed by Western blot probed with anti-GFP monoclonal antibodies. Caspase cleavage released the monomeric GFP as indicated by the arrow. pGDB expresses a previously described BFP-GFP hybrid protein linked by a DEVD containing peptide ('GFP'-GSGSGSDEVDGSGSGS-'BFP' (SEQ ID NO:10), wherein the caspase cleavage site is in between V and D) used as a reporter of intracellular caspase activity [Xu et al., Nucl. Acids Res. 26:2034-2035 (1998)]. When caspases 2, 3, or 7 are activated, they specifically cleave appropriately accessible substrates at the DEVD peptide [Cryns et al., Cell 82:349-352 (1995)] and release GFP/BFP monomers. Western blot analysis using anti-GFP monoclonal antibody indicated that ectopic expression of Apop3 activated caspases to cleave the DEVD linker peptide, releasing GFP/BFP monomer (data not shown). A broad-spectrum inhibitor of caspases, zVAD-fmk [Zhu et al., FEBS Lett. 374:303-308 (1995)], inhibited the caspase activity triggered by Apop3 expression (data not shown). This result confirmed that the cleavage of the chimeric protein was due to caspase activation (data not shown). Apop3-induced apoptosis could also be inhibited by CrmA (data not shown). N-terminal deletion mutants Apop3(82-518), Apop3(287-518) and Apop3(K50D) were still able to activate the caspase activity (data not shown). Deletion of the C-terminal region of Apop3, Apop3(1-251) and Apop3(1-436), completely abrogated caspase activation (data not shown). These results indicate again that Apop3's kinase activity is not required for activation of caspases and cell death. Since the C-terminal domain (aa 437-518) of Apop3 is not required for the binding of Apop3 to RIP, this region may be involved